

# Th2 cytokines increase and stimulate B cells to produce IgG4 in idiopathic membranous nephropathy

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## Th2 cytokines increase and stimulate B cells to produce IgG4 in idiopathic membranous nephropathy.

**Background.** The predominant deposition of IgG4 in idiopathic membranous nephropathy indicates that its presence characterizes the systemic immune response of the disease.

**Methods.** We analyzed the expressions of CD3, CD19, CD4, and CD8 on peripheral blood mononuclear cells (PBMCs) by flow cytometry, and the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interferon (IFN)- $\gamma$ , interleukin (IL)-4, IL-10, and IL-13 mRNAs in PBMCs using real-time reverse transcription-polymerase chain reaction (RT-PCR) in 14 patients with idiopathic membranous nephropathy and 14 normal control donors. The levels of IgG subclasses in the B-cell culture supernatant in the presence or absence of cytokines were quantified by enzyme-linked immunosorbent assay (ELISA).

**Results.** Idiopathic membranous nephropathy patients showed an increased CD4<sup>+</sup>/CD8<sup>+</sup> ratio, although the numbers of peripheral T and B cells were comparable to those of the normal control group. IL-10 and IL-13 mRNA expression levels increased in the idiopathic membranous nephropathy group. The levels of spontaneous production of each IgG subclass by B cells were identical in the two groups. In the presence of Th2 cytokines, B cells from several individuals of the idiopathic membranous nephropathy group augmented the production of IgG4. When the individual levels of each IgG subclass in the presence of cytokines were compared with those in the absence of cytokines in each sample, a significant increase in the production of IgG4 in the presence of IL-4 was observed in the idiopathic membranous nephropathy group.

**Conclusion.** These results indicate that the altered functions of T cells to produce Th2 cytokines and the increased production of IgG4 by B cells in response to these cytokines characterize the immune response in idiopathic membranous nephropathy.

**Key words:** idiopathic membranous nephropathy, peripheral lymphocytes, cytokines, IgG4.

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Research into the pathogenesis of idiopathic membranous nephropathy has centered primarily on antigen identification. The understanding of the disease has been greatly advanced by the Heymann nephritis model, a rat experimental model of idiopathic membranous nephropathy. In this experimental model, megalin, a common component in the tubular and glomerular epithelial cells, was identified as a target antigen [1–3]. Furthermore, this model demonstrated the critical role of C5b-9-induced podocyte injury [4, 5] and podocyte response [6–9], which result in proteinuria. However, megalin does not exist in human podocytes, and the target antigen in human idiopathic membranous nephropathy has not been identified. Although the rat Heymann nephritis model provided the molecular mechanisms of proteinuria, the antigen-specific immune response in this model is not applicable to the corresponding human disease. Studies using human samples are required to elucidate the nephritogenic immune responses in idiopathic membranous nephropathy.

Some attempts to define the pathogenesis of idiopathic membranous nephropathy have been made using biopsy samples or the peripheral blood of patients, and several immunologic abnormalities have been described. For example, previous studies showed an imbalance between the CD4<sup>+</sup> and CD8<sup>+</sup> subsets [10–15], diminished synthesis of immunoglobulin by peripheral lymphocytes [16], decreased suppressor T-cell function [11, 13, 17], and predominance of the Th2 immune response [18, 19] in idiopathic membranous nephropathy. Furthermore, several reports have shown that IgG4 predominates in the renal biopsies of idiopathic membranous nephropathy [20–23] and this predominance is not observed in secondary or lupus-associated membranous nephropathy. Moreover, Doi et al [24] showed that IgG4 is overrepresented in the circulating immune complex in idiopathic membranous nephropathy. These observations suggest that immune response in idiopathic membranous nephropathy may be characterized by the presence of IgG4. In this study, to understand the immune responses in idiopathic membranous nephropathy, we focused on the production and regulation of IgG4. We analyzed the subpopulations

and functions of peripheral lymphocytes, including the *in vitro* production of IgG subclasses by B cells and their responses to the cytokines.

## METHODS

### Subjects

Venous blood samples were obtained 2 or 3 days after the biopsy, when the diagnosis of idiopathic membranous nephropathy was highly indicated by a routine immunofluorescence study, which was performed 2 days after the biopsy. Among these, 14 patients were selected to participate in the study on the basis of the diagnosis of idiopathic membranous nephropathy confirmed by light and electron microscopy. These patients were confirmed to have no systemic disease that may cause secondary membranous nephropathy by clinical and laboratory examinations. Their clinical and laboratory parameters were nine males and five females, mean age  $60.4 \pm 15.0$  years, mean urinary protein excretion  $2.5 \pm 3.7$  g/day, mean serum total protein  $5.8 \pm 1.2$  g/dL, mean serum albumin  $3.0 \pm 1.1$  g/dL, mean serum creatinine  $0.9 \pm 0.4$  mg/dL, and mean creatinine clearance  $79.5 \pm 39.5$  mL/min. The histologic stage was classified into four stages according to the electron microscopic findings. One patient was classified as stage I, eight patients as stage II, four patients as stage III, and one patient as stage IV. For the normal controls, venous blood samples were obtained from 14 age-matched (mean  $54 \pm 19$  years), healthy volunteers, whose renal function and urinary sediment were proved to be normal in routine medical examinations. None of the patients had received any immunosuppressive drugs, including steroids, at the time of sample collection. Informed consent was obtained from all patients and healthy volunteers.

### Glomerular IgG subclasses

Glomerular IgG subclass deposition was examined by direct immunofluorescence microscopy, using fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies as follows: clone HP-6069 (Zymed Laboratories, San Francisco, CA, USA) (anti-IgG1), HP-6014 (Sigma Chemical Co., St. Louis, MO, USA) (anti-IgG2), HP-6047 (Zymed Laboratories) (anti-IgG3), and HP-6025 (Sigma Chemical Co.) (anti-IgG4). The fluorescence intensity of glomerular staining was graded as follows: negative, 0; very weak, 0.5; weak, 1; moderate, 2; and strong, 3, and scored for each specimen. The results are expressed as the frequency of positive biopsies and the intensity of fluorescence for each IgG subclass.

### Cytofluorometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque gra-

dient. PBMCs were stained with the following combinations of monoclonal antibodies: anti-CD3/FITC (BD PharMingen, San Diego, CA, USA) and anti-CD 19/PE (BD PharMingen), to analyze T and B lymphocytes; anti-CD3/Cy (BD PharMingen), anti-CD8/FITC (BD PharMingen), and anti-CD4/PE (BD PharMingen), to analyze the CD4<sup>+</sup> and CD8<sup>+</sup> subsets of T lymphocytes. Flow cytometry results were analyzed using Cell Quest software.

### RNA preparation and cDNA synthesis

Total RNA was prepared from peripheral whole blood using an RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with 5 µg of total RNA using an oligo(dT) primer (Invitrogen, Carlsbad, CA, USA), deoxynucleoside triphosphate (dNTP) (Promega, Madison, WI, USA), RNase inhibitor (Invitrogen), and a SuperScript RT kit (Life Technologies, Inc., Gaithersburg, MD, USA) containing 5 × reverse transcription (RT) buffer, 0.1 mol/L dithiothreitol (DTT) and reverse transcriptase.

### Cytokine mRNA levels in PBMCs

Real-time quantitative polymerase chain reaction (PCR) was carried out using a Light Cycler Instrument, and the DNA binding dye SYBR Green I for the detection of PCR products. The target molecules were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interferon (IFN)- $\gamma$ , interleukin (IL)-4, IL-10, and IL-13. Reaction mixtures for the real-time PCR had a final volume of 20 µL consisting of 10 µL of cDNA, 2 µL of commercially synthesized primers (LightCycler™ Primer Set, Roche GmbH, Heidelberg, Germany), 2 µL of Faststart SYBR Green I (Roche GmbH), and 6 µL of distilled water. Amplification conditions were 10 minutes at 95°C and 35 cycles of 10 seconds at 95°C, 10 seconds at 68°C, and 16 seconds at 72°C. The quantification of gene expression was performed using the amplification standards contained in the kit. Cytokine gene expression was expressed in relative copy number normalized against GAPDH mRNA expression level.

### Enzyme-linked immunosorbent assay (ELISA) of IgG subclasses

IgG subclass concentrations in supernatants were quantitated by ELISA as described previously [22]. Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with one of the human IgG subclass specific mouse monoclonal antibodies (Calbiochem Corp., La Jolla, CA, USA) at 5 µg/mL for HP6069 (anti-IgG1), HP6002 (anti-IgG2), and HP6025 (anti-IgG4), and at 2.5 µg/mL for HP6047 (anti-IgG3). Samples were then added to the microtiter plates. The assays were developed with

**Table 1.** Glomerular IgG subclass distribution

	IgG1	IgG2	IgG3	IgG4
Number of positive biopsies (%)	3 (27.3)	2 (18.2)	1 (9.1)	11 (100)
Intensity of fluorescence <sup>a</sup> (mean $\pm$ SD)	0.18 $\pm$ 0.44	0.18 $\pm$ 0.40	0.09 $\pm$ 0.30	1.09 $\pm$ 0.49

Glomerular IgG subclass distribution was studied in 11 patients with idiopathic membranous nephropathy.

<sup>a</sup>Intensity of fluorescence is expressed as a score.

alkaline phosphatase (AP)-conjugated mouse polyclonal antihuman IgG antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). For each assay, the standard curve was established using the human standard serum NOR-01/91 (Nordic Immunological Laboratories, Tilburg, The Netherlands).

### Isolation of peripheral B cells

B cells were isolated by negative selection from PBMCs using a cross-linking reagent (RosetteSep<sup>TM</sup> antibody cocktail for human B cells) (Stemcell Technologies Inc., British Columbia, Canada), which is a mixture of mouse and rat monoclonal antibodies. These antibodies are bound in bispecific antibody complexes, which are directed against cell surface antigens for human hematopoietic cells (CD2, CD3, CD16, CD36, and CD56) and glycophorin A on red blood cells. The cell fractions obtained contained >95% CD19<sup>+</sup> B cells as estimated by flow cytometry.

### In vitro production of IgG subclasses by B cells

B cells were cultured at a concentration of  $2 \times 10^5$  cells/well in 96-well plates in a final volume of 0.2 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/L glutamine, 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. On the basis of a preliminary kinetic experiment showing that isolated B cells produce IgG without any stimulation and the levels of IgG subclasses reach the maximum after 7 days of incubation (data not shown), the supernatants were harvested on day 7 of incubation and the levels of IgG subclasses were measured by ELISA. B cells were also cultured in the presence of 50 ng/mL of IFN- $\gamma$  (R&D Systems, Abington, UK), IL-4 (Geneva Biochemical Research Institute, Geneva, Switzerland), IL-10 (R&D Systems), or IL-13 (R&D Systems) to determine the effect of these cytokines on IgG subclass production. The concentrations of cytokines were determined on the basis of a preliminary experiment using isolated B cells from healthy volunteers showing that these cytokines had no dose-dependent effects on IgG subclass production in the range of 0.5 to 50 ng/mL. To assess the influence of cytokines on the IgG subclasses, the increase ratio of cytokine-stimulated IgG subclass synthesis by B cells was calculated using the following formula for individual samples: increase ratio = (IgG subclass level in the

presence of cytokines)/(IgG subclass level in the absence of cytokines).

### Statistical analyses

Comparisons were done using the Mann-Whitney U test, and correlations were tested by Spearman rank correlation, performed with StatView 5. *P* values greater than 5% were considered insignificant.

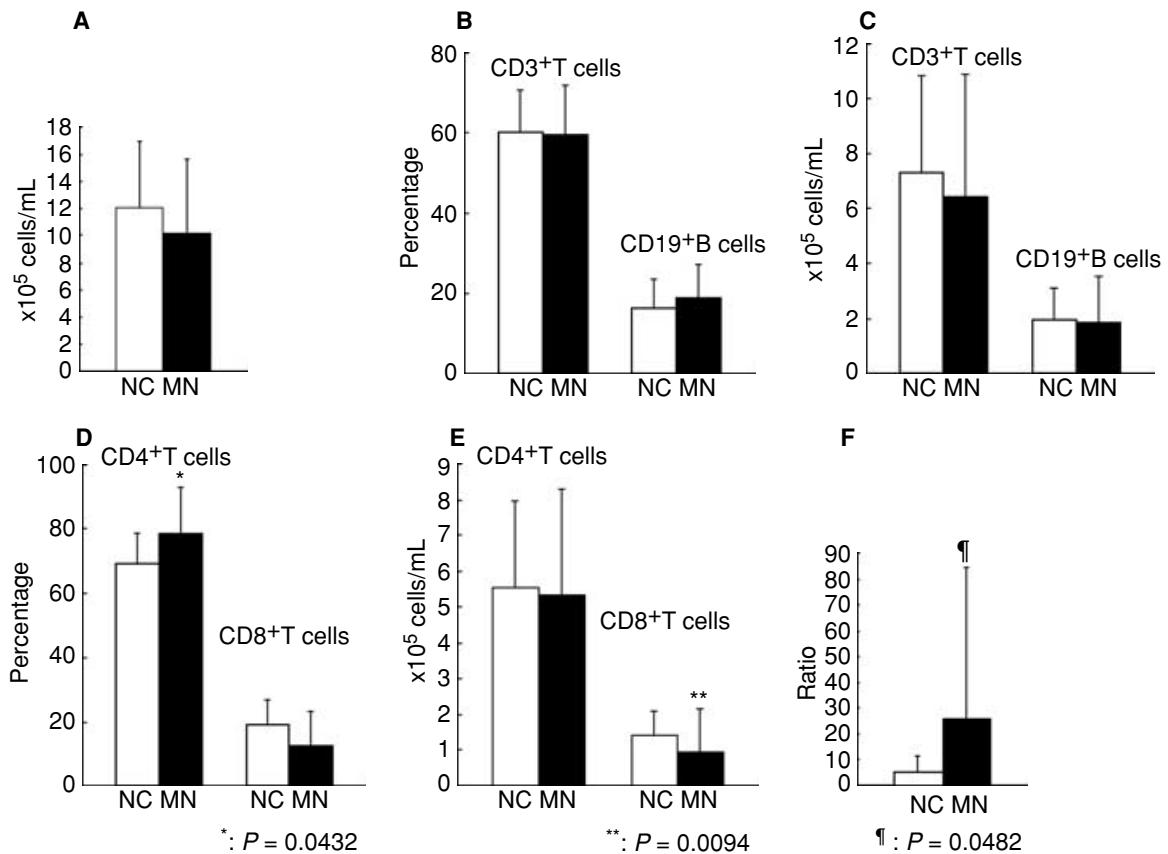
## RESULTS

### IgG4 was the major glomerular IgG subclass in idiopathic membranous nephropathy

Glomerular IgG subclasses were examined in 11 patients with idiopathic membranous nephropathy. As shown in Table 1, IgG4 was the major glomerular IgG subclass, both in the fluorescence intensity and in the frequency of positivity. These results were consistent with those of previous reports. There was no correlation between the fluorescence intensity of the staining of IgG4 and the ultrastructural stages of glomerular lesions.

### Percentage of CD4<sup>+</sup> T cells increased in patients with idiopathic membranous nephropathy

According to the results for the glomerular IgG subclasses, IgG4 subclass antibodies seem to be pivotal in the immunopathogenesis of idiopathic membranous nephropathy. To study the possible alterations in the production of IgG4 and its regulation, we first examined the peripheral lymphocytes by flow cytometry. The numbers of peripheral lymphocytes, T cells, and B cells were not significantly different between the two groups (Fig. 1A to C). However, in the idiopathic membranous nephropathy group, fluorescence-activated cell sorter (FACS) analysis showed a significant increase in the percentage of CD4<sup>+</sup> T cells, and a significant decrease in the number of CD8<sup>+</sup> T cells, resulting in a marked increase in the CD4<sup>+</sup>/8<sup>+</sup> ratio (Fig. 1D to F). These alterations in the lymphocyte subpopulations did not correlate with the levels of urinary protein, serum total protein, or albumin. These results indicate that the elevated CD4<sup>+</sup>/8<sup>+</sup> ratio in patients with idiopathic membranous nephropathy was not a consequence of proteinuria.



**Fig. 1. Peripheral lymphocyte subpopulations in patients with idiopathic membranous nephropathy (MN) and normal controls (NC).** The numbers of lymphocytes were calculated based on the results of flow cytometry. (A) Numbers of the peripheral lymphocytes. (B) Percentages of CD3<sup>+</sup> T cells and CD19<sup>+</sup> B cells. (C) Numbers of CD3<sup>+</sup> T cells and CD19<sup>+</sup> B cells. (D) Percentages of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. (E) Numbers of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. (F) CD4<sup>+</sup>/8<sup>+</sup> ratio. Each column and bar represents the mean and 1 SD. The numbers of lymphocytes, both T and B cells were identical in the two groups. The idiopathic membranous nephropathy patients showed an increased percentage of CD4<sup>+</sup> T cells, and a decreased number of CD8<sup>+</sup> T cells, resulting in a high CD4<sup>+</sup>/8<sup>+</sup> ratio.

### IL-10 and IL-13 mRNA expression levels in PBMCs increased in idiopathic membranous nephropathy

Because cytokines have an effect on the production of IgG subclasses, patients with idiopathic membranous nephropathy may exhibit distinct cytokine patterns. Therefore, we quantified the mRNA levels of IFN- $\gamma$ , IL-4, IL-10, and IL-13 in PBMCs using real-time RT-PCR. There were no differences in the levels of IFN- $\gamma$  and IL-4 mRNAs between the idiopathic membranous nephropathy group and the normal control group (Fig. 2A and B). In contrast, the relative copy numbers of IL-10 and IL-13 were significantly higher in the idiopathic membranous nephropathy group than in the normal control group (Fig. 2C and D).

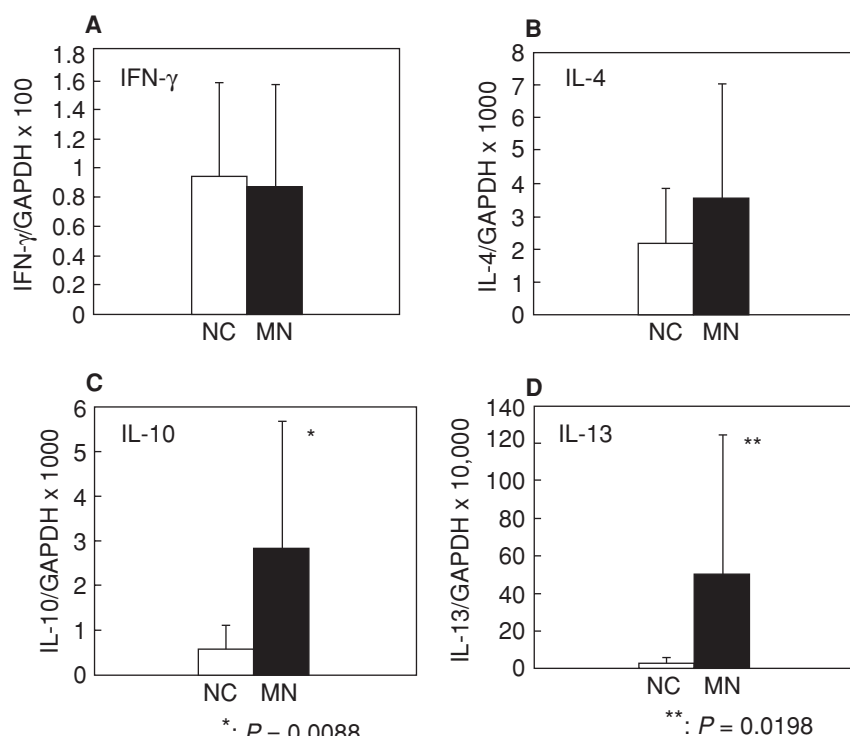
### In vitro production of IgG subclasses by B cells

B cells are responsible for the production of immunoglobulins, and thus the function of B cells to produce IgG subclasses may be altered in patients with idiopathic membranous nephropathy. To investigate this possibility, we analyzed the levels of IgG subclasses in

the culture supernatants of isolated B cells. As shown in Fig. 3A, unstimulated B cells produced measurable amounts of IgG subclasses in both groups. Although B cells from three patients with idiopathic membranous nephropathy showed a higher spontaneous production of IgG4, there was no significant difference in the concentrations and percentage of each IgG subclass, including IgG4, between the idiopathic membranous nephropathy group and the normal control group. The levels of total IgG were also identical in the two groups. Without stimulation, B cells from patients with idiopathic membranous nephropathy showed no significant change in the production of IgG subclasses.

### Correlation between immunologic alterations and glomerular depositions of IgG4

Alterations in immune response may lead to the glomerular deposition of IgG4 in membranous nephropathy. Therefore, we analyzed the association between immunologic alterations and the fluorescence intensity of staining of glomerular IgG4 in each patient. There was no



**Fig. 2. Cytokine mRNA levels in peripheral blood mononuclear cells (PBMCs).** Cytokine mRNA levels were quantified using real-time reverse transcription-polymerase chain reaction (RT-PCR) and results were expressed in relative copy numbers normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. (A) Interferon (IFN)- $\gamma$ . (B) Interleukin (IL)-4. (C) IL-10. (D) IL-13. Each column and bar represents the mean and 1 SD. The levels of Th2 cytokines IL-10 and IL-13 increased in the idiopathic membranous nephropathy (MN) group. NC is normal controls.

correlation between the mRNA expression levels of cytokines and the concentrations or ratios of IgG subclasses in patients with idiopathic membranous nephropathy. Furthermore, the mRNA expression levels of cytokines and the concentrations or ratios of IgG subclasses did not correlate with the fluorescence intensity of staining of glomerular IgG4 in these patients.

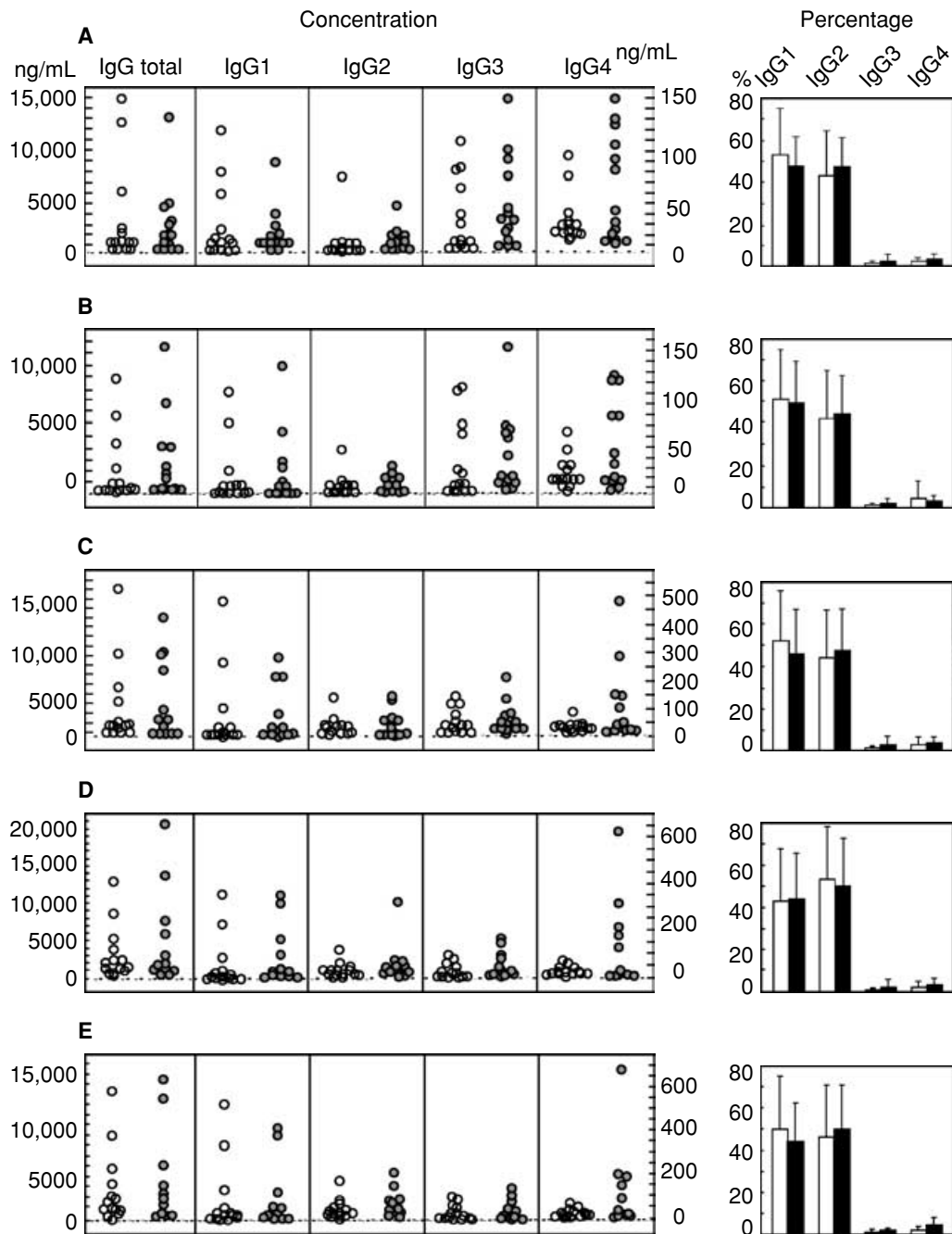
#### IL-4 enhances in vitro production of IgG4 by B cells in idiopathic membranous nephropathy

The above results showing that cytokine patterns differ in the idiopathic membranous nephropathy patients raise the possibility that these cytokines affect the production of IgG subclasses by B cells. To investigate the influence of cytokines on the production of IgG subclasses, we cultured B cells in the presence of 50 ng/mL of IFN- $\gamma$  ( $N = 13$ ), IL-4 ( $N = 13$ ), IL-10 ( $N = 12$ ), or IL-13 ( $N = 11$ ). Figure 3B shows that the presence of IFN- $\gamma$  had no effect on the production of IgG subclasses in either group. As shown in Figure 3C, D, and E, B cells from some of the idiopathic membranous nephropathy patients produced increased amounts of IgG4 in the presence of IL-4, IL-10, or IL-13. However, no significant difference was observed in the concentrations of IgG4 and the other three IgG subclasses in the presence of these cytokines between the two groups. Moreover, a comparison of the levels of each IgG subclass in the presence and absence of cytokines showed no significant difference in both groups. To evaluate the influence of cytokines in more

detail, we compared the levels of IgG subclasses in the presence and absence of cytokines in single individuals, and expressed the results as increase ratio. When the increase ratio of each IgG subclass was compared between the two groups, the mean increase ratios of IgG4 in the presence of IL-4 were significantly higher in the idiopathic membranous nephropathy group, although those of IgG1, IgG2, and IgG3 were not significantly different (Fig. 4). In the presence of IFN- $\gamma$ , IL-10, or IL-13, no significant difference was observed in the increase ratio of IgG subclasses between the two groups. These results indicated that IL-4 enhanced the in vitro production of IgG4 by B cells in idiopathic membranous nephropathy.

#### DISCUSSION

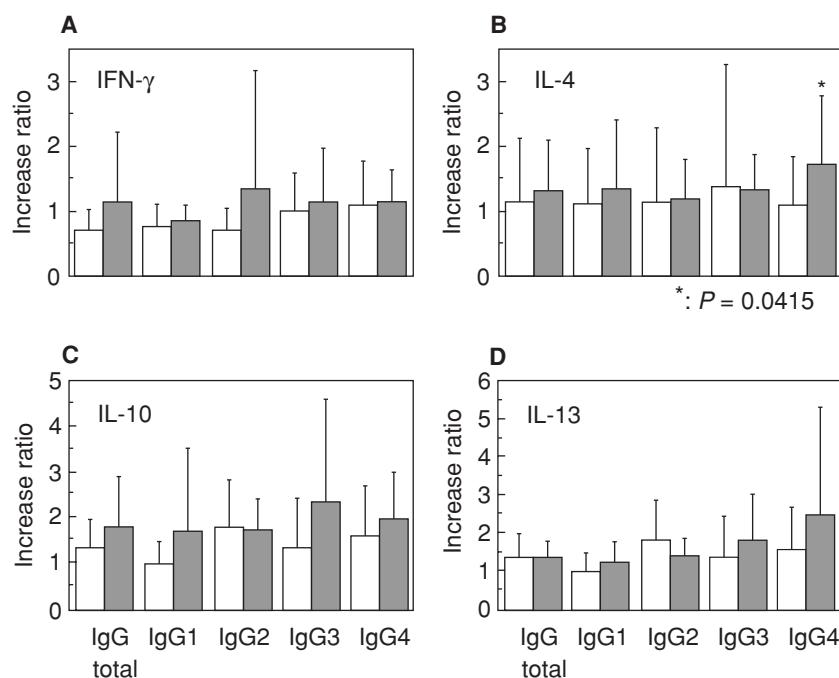
The precise nature of the disease-initiating antigen in human idiopathic membranous nephropathy remains unclarified, despite the fact that the rat Heymann nephritis model has afforded insight into the pathogenesis of the disease. On the basis of the findings that the number of IL-10- or IL-4-secreting T cells increases in idiopathic membranous nephropathy [18, 19] and that IgG4, which is considered to be a human homologue of murine Th2-type subclass IgG1, predominates in glomerular IgG subclasses in idiopathic membranous nephropathy, it has been suggested that the disease-initiating antigen triggers Th2-type immune responses in idiopathic membranous nephropathy. Although the validity of the Th1/Th2



**Fig. 3. IgG subclass production by B cells.** B cells were cultured in the absence (A) or presence of cytokines. (B) Interferon (IFN)- $\gamma$ . (C) Interleukin (IL)-4. (D) IL-10. (E) IL-13. After 7 days of incubation, the levels of IgG subclasses in the supernatant were quantified. For the concentrations, the scales on the left side are for total IgG, IgG1, and IgG2, and the scales on the right side are for IgG3 and IgG4. Percentages are expressed as the mean and 1 SD. Open circles and bars represent normal controls, and closed circles and bars represent idiopathic membranous nephropathy. Although some B cells from idiopathic membranous nephropathy patients augmented the production of IgG4 in response to IL-4, IL-10, or IL-13, there was no significant difference in the concentration and percentage of each IgG subclass between the two groups.

paradigm in humans is not universally accepted, the immune responses in idiopathic membranous nephropathy have been simplified as “Th2 predominance,” and no further attempts have been made to clarify the association between these cytokines and the production of IgG4. Therefore, in this study, we analyzed the functions of pe-

ripheral lymphocytes in patients with idiopathic membranous nephropathy, to define the characteristics of the immune response, which may be useful to elucidate the causal antigen of the disease. We observed an increased CD4<sup>+</sup>/CD8<sup>+</sup> ratio, higher levels of IL-10 and IL-13 mRNAs in PBMCs, and increased in vitro production of



**Fig. 4.** To assess the effect of cytokines on the production of IgG subclasses in individual samples, the increase ratio was calculated. (A) Interferon (INF)- $\gamma$ . (B) Interleukin (IL)-4. (C) IL-10. (D). IL-13. Columns and bars indicate the mean and 1 SD of increase ratios for each group. The mean increase ratio of IgG4 in the presence of IL-4 in the idiopathic membranous nephropathy group was significantly higher than that in the normal control group.

IgG4 by B cells in the presence of IL-4, in the patients with idiopathic membranous nephropathy.

Previous studies of the T-cell subsets in idiopathic membranous nephropathy demonstrated an increased CD4<sup>+</sup>/CD8<sup>+</sup> ratio [10–15], which was consistent with our findings. The observation that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio returned to the normal level in the persistent stage or in the complete remission of the disease [14, 15] raises the possibility that these alterations of T-cell subsets are the consequences of proteinuria. Our finding that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio did not correlate to the amount of urinary protein indicates that this imbalance of T cells in idiopathic membranous nephropathy patients was not secondary to proteinuria. However, since abnormalities of T-cell subsets are also observed in patients with IgA nephropathy [10, 12, 13], we cannot consider this imbalance as a pathologic status specific to idiopathic membranous nephropathy.

It is crucial to determine whether these alterations in T-cell subsets are associated with functional changes in patients with idiopathic membranous nephropathy. Several studies showed the defective [12, 13, 17] concanavalin A (Con A)-inducible functions of suppressor T cells in idiopathic membranous nephropathy. Because whole PBMCs were used in these studies, the impaired suppressor cell function may not reflect a functional change, but could be a result of the decreased number of CD8<sup>+</sup> T cells. The assessment of cytokine patterns is one of the approaches to determining the functions of T cells. Hirayama et al [18], using flow cytometry, found a decreased number of IL-2-positive CD4<sup>+</sup> T cells and an increased number of IL-10-positive CD4<sup>+</sup> T cells in patients with idiopathic membranous nephropathy, although there was

no significant difference in the number of IFN- $\gamma$ -positive or IL-4-positive CD4<sup>+</sup> T cells. In contrast, Masutani et al [19] demonstrated the up-regulation of IL-4 production by CD4<sup>+</sup> T cells, using the same method. In the present study, we used quantitative real-time RT-PCR to assess the cytokine patterns, a method which has been proved to be sensitive [25] for IL-4 detection, which is difficult in humans. Thus, we were able to determine cytokine patterns without any stimulation of PBMCs and observed increased expression levels of IL-10 and IL-13 mRNAs in idiopathic membranous nephropathy patients, although those of IFN- $\gamma$  and IL-4 were not significantly different from those in the normal control group. In contrast to Masutani et al [19], we could not demonstrate a significant change in IL-4 mRNA expression level in the idiopathic membranous nephropathy group. The discrepancy may be due to the method of detecting IL-4. Ekerfelt et al [25], who compared three assays [(ELISPOT), ELISA, and real-time RT-PCR] that can detect IL-4 showed that mRNA expression levels do not correlate with the secretion of IL-4 [25]. Accordingly, our results of the expression levels of IL-4 mRNA were different from the results of the study by Masutani et al, in which IL-4 production was analyzed. Moreover, since the amount of blood samples obtained was not sufficient to sort out CD4<sup>+</sup> T cells, we used whole PBMCs for RNA extraction. For this reason, our results did not precisely reflect the functional changes of T cells. Nevertheless, our observations, together with the two previous findings indicate that idiopathic membranous nephropathy results from the predominance of Th2-type immune response.



There have been two studies that analyzed the *in vitro* production of IgG, but not its subclasses, using whole PBMCs from idiopathic membranous nephropathy patients [13, 16], which may interfere with the production of IgG by B cells. We used purified B cells to closely evaluate the functions of B cells, avoiding the interference of other peripheral mononuclear cells. The functional tests showed that B cells augmented IgG4 synthesis when stimulated by Th2 cytokines in some individuals in the idiopathic membranous nephropathy group, although the spontaneous production of all four IgG subclasses was similar to that of the normal control group. Human peripheral B cells can be divided into two distinct populations: naïve B cells (60%) and memory B cells (40%) [26], with only the latter group being able to produce IgG when stimulated *in vitro* [27]. It is unlikely that class switching occurred in our culture system, since CD40 stimulation with cytokines induces a differentiation blockade of naïve B cells [27] and a prolonged CD40 stimulation is necessary for the naïve B cells to switch to CD27-positive memory B cells [28]. Accordingly, one possible explanation for our results is that although the number of peripheral B cells did not change, as confirmed by flow cytometry, the percentage of memory or previously activated circulating B cells increased in the patients with idiopathic membranous nephropathy, the cells being ready to produce IgG4 in response to Th2 cytokines. Furthermore, the expression levels of cytokine receptors on B cells can account for the responsiveness to the cytokines. B cells would respond highly to IL-4, when the IL-4 receptor is up-regulated, albeit the expression level of IL-4 mRNA was not significantly increased. To confirm these hypotheses, further analysis of B cell surface markers, for example, CD20 (marker of activated B cells), CD27 (marker of memory B cells) [29], and CD124 (IL-4 receptor), will be necessary. Recent studies that show an increased percentage of CD20-positive B cells in patients with idiopathic membranous nephropathy [30, 31] support our hypotheses.

The elucidation of the target antigen is of the utmost importance in terms of understanding the pathogenesis of human idiopathic membranous nephropathy. Neutral endopeptidase is one of the antigens shared by the brush border and podocytes that are involved in the formation of immune deposits in animal models; this protein is also expressed on human podocytes. Recently, Debiec et al [32] have reported a case of antenatal idiopathic membranous nephropathy due to antineutral endopeptidase antibodies, which were transferred from a mother who was deficient in neutral endopeptidase and was thought to be immunized against the antigen at the time of an earlier miscarriage. However, this antigen-antibody response is not generally applicable to the pathogenesis of idiopathic membranous nephropathy. The functional alterations of peripheral B cells to produce IgG4 in the presence of cy-

tokines observed in the present study provide some clues to the characteristics of the disease-specific antigen in idiopathic membranous nephropathy. The IgG4 subclass predominance has been observed for autoantibodies to thyroglobulin and microsomes in Hashimoto's thyroiditis [33], autoantibodies to the muscle-specific kinase in myasthenia gravis [34], autoantibodies in pemphigus vulgaris [35], and autoantibodies to glomerular basement membrane in Goodpasture's syndrome [36]. Thus, in these organ-specific autoimmune diseases, autoantibodies to organ components often belong to the IgG4 subclass. Accordingly, a strong candidate for the disease-specific antigen in idiopathic membranous nephropathy may be a molecule expressed in the glomerulus, or more exactly, in podocytes. On the other hand, IgG4 is produced after recurrent stimulation with antigens [37]: blocking antibodies following specific allergen therapy belong mainly to this subclass [38] and the shift to IgG4 is noted in chronic helminth infection [39]. Consequently, chronic infections, in which antibodies of the IgG4 subclass generated in response to an infectious agent cross-react with a component of podocytes, can initiate idiopathic membranous nephropathy. However, to date, there is no evidence of chronic infections in idiopathic membranous nephropathy. Isotype switching to IgG4 is dependent not only on antigenic stimulation, but also on regulatory factors produced by T cells. As mentioned already, the evidence for Th2 predominance in idiopathic membranous nephropathy observed in this study is in accordance with the importance of IgG4 in this disease.

T cells from thymic precursors initially express both the CD4 and CD8 coreceptors, and then become either CD4 or CD8 single-positive thymocytes. The precise mechanism of this coreceptor-defined lineage remains unknown. Interestingly, a more recent study showed that the CD4 versus CD8 lineage of immature thymocytes is controlled by the coreceptor-influenced duration of the initial T-cell receptor signaling. Long-lasting signals would be necessary to induce the CD4 program [40]. Although the results from the *in vitro* experiment are not directly applicable to our observation of peripheral T cells, they may explain the increased CD4<sup>+</sup>/8<sup>+</sup> ratio, further suggesting the presence of a prolonged stimulation in idiopathic membranous nephropathy.

Our study demonstrated the predominance of IgG4 in renal biopsies and showed that the functions of both T and B cells are altered in idiopathic membranous nephropathy, resulting in Th2 predominance and increased production of IgG4 by B cells in response to these cytokines. Although the precise nature of the disease-initiating antigen in idiopathic membranous nephropathy remains unknown, our findings provide the characteristics of the disease-specific immune response, which has implications for a new therapeutic approach to the disease.



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